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Translation of the Genome of a Ribonucleic Acid Bacteriophage

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INTRODUCTION

The discovery that the genome of certain small ribonucleic acid (RNA)-containing viruses serves directly as a template for the biosynthesis of viral proteins opened the way for detailed study of the translation of natural messenger RNA (mRNA). Ease of preparation of highly purified coliphage RNA, coupled with intensive exploration of protein synthesis in extracts of *Escherichia coli*, the natural host for this group of viruses, made such an homologous system favored material for investigating the mechanism of protein synthesis. With the polycistronic phage RNA as messenger, fundamental discoveries have been made concerning the initiation, elongation, and termination steps of protein synthesis, the function of ribosomes, and the mode of action of various antibiotics. As understanding of the growth cycle and genetics of the RNA coliphages developed, biochemical experiments became possible which clarified such general phenomena as polarity, suppression, and translational regulation. Most recently, much of the nucleotide sequence of the phage RNA has been determined, providing fresh insights or solid confirmation of previous hypotheses regarding various aspects of the translational process. Taken as a whole, the work on RNA coliphages by many investigators over the past 10 years has resulted in the most comprehensive picture so far available of the molecular biology of a single organism.

In this review we attempt to summarize, evaluate, and interrelate observations on the translation of the RNA bacteriophage genome, with emphasis on regulation at the translational level. For even with this relatively simple biological replicating system, regulation of gene expression is evident both in infected cells and in cell extracts programmed with the viral RNA, and it is now possible to describe some of these control mechanisms in considerable molecular detail.

GENERAL PROPERTIES OF RNA COLIPHAGES

The male-specific RNA coliphages consist of three physically (130) and serologically (97) distinguishable groups. One group includes f2, MS2, M12, R17, and R23; the second group includes Qβ and related phages; the third group includes fr, f4, and β (for a more extensive list, see reference 209). The relatedness of viruses of the f2 group, which have been used most frequently for studies of protein synthesis, is evident from immunological assays (97, 161), physical studies on the virions (44, 178), chemical analyses of the coat protein (37, 100, 211, 220) and RNA (28, 57, 118, 129, 191, 192), and template specificity of the viral RNA synthetase (120). In view of these similarities, we generally make no distinction in this review among particular members of the f2 group. Coliphage Qβ, which also has been studied intensively, is slightly larger (137) and possesses little or no antigenic similarity (97, 137) or overall nucleotide sequence homology (138, 218, 219) with the f2 group, and the Qβ RNA synthetase is inactive with MS2 RNA (58, 120). Nevertheless, Qβ is analogous to the other RNA coliphages in structure and intracellular development, nucleotide sequences of limited regions of its RNA are strikingly similar to sequences in R17 RNA (2, 48), and there is extensive homology in the amino acid sequences of Qβ and f2 coat proteins (94). These similarities suggest the possibility of a common evolutionary ancestor for all of the RNA coliphages.

Although the biology of the various male-specific RNA coliphages is quite similar, there are some important differences. Notable among these are the marked inhibition of host RNA synthesis which occurs only during infection by R17 or R23 (32, 49, 73, 77, 78, 208, 210) and synthesis of an unusual fourth viral protein in Qβ-infected cells (*see below*). In addition, it has been claimed that Qβ (but not MS2) induces a change in proline transfer RNA (tRNA) in infected cells (75). However, the evidence for this potentially interesting alteration of tRNA is indirect, and there is at present no convincing demonstration of tRNA modification following RNA phage infection.

Structure of Phage Particles

The icosahedral phage particle, which is about 25 nm in diameter (40, 67, 116, 178) and has a mass of 3.6×10^6 amu (44, 178), contains two species of protein and one molecule of RNA. The major structural protein (coat protein) accounts for about 70% of the mass of the particle; the minor structural protein (A protein or maturation protein) is probably present in only one copy per virion (136, 173). In Qβ particles another minor protein is also present (*see below*). There is some evidence for an asymmetric projection on the surface of the phage particle (40), but whether this is related to the presence of maturation protein is not known.

The nucleic acid component of the virus is a single-stranded, linear RNA molecule of about 1.1×10^6 to 1.3×10^6 daltons in the case of the f2 group (10, 44, 165, 178), whereas Qβ RNA is somewhat larger (10). The genome of the former group thus contains about 1,200 potential codons. The nucleotide sequences of large stretches of the RNA have been determined, and some of the currently available results are shown in Fig. 1 and Fig. 2. In addition to identifying nucleotide sequences coding for viral polypeptides (38, 81, 129), these studies have also identified untranslated regions between the cistrons (Fig. 2) and at the 5′ and 3′ ends of the RNA (8, 23, 24, 28, 48, 101).

There are extensive regions of secondary structure in the RNA, revealed by hyperchromicity on heating (178), sedimentation properties (44), limited reactivity with formaldehyde at low temperatures (119), and the initial kinetics of hydrolysis by alkali or ribonuclease (180). Nucleotide sequence analyses confirm that many regions of the molecule are capable of base-pairing either with adjacent sequences or with more distant regions (Fig. 1). The specific base-pairing shown in the figure is hypothetical, and more than one stable conformation may be possible. In any case, there is clear evidence that the conformation of phage RNA plays an important role in translation. Besides regulating the messenger activity of the RNA, as described in detail below, the extensive secondary structure may also account for the stability of the molecule in vivo.

Infection and Phage Development

The developmental cycle of an RNA coliphage requires about 40 min at 37 C, during which time about 10^4 progeny virus particles are formed per infected cell. Infection is initiated by adsorption of a phage particle to the side of an F pilus of a male strain of *E. coli* (12, 26). Virions lacking maturation protein are unable to adsorb to pili (112, 197). After ad-

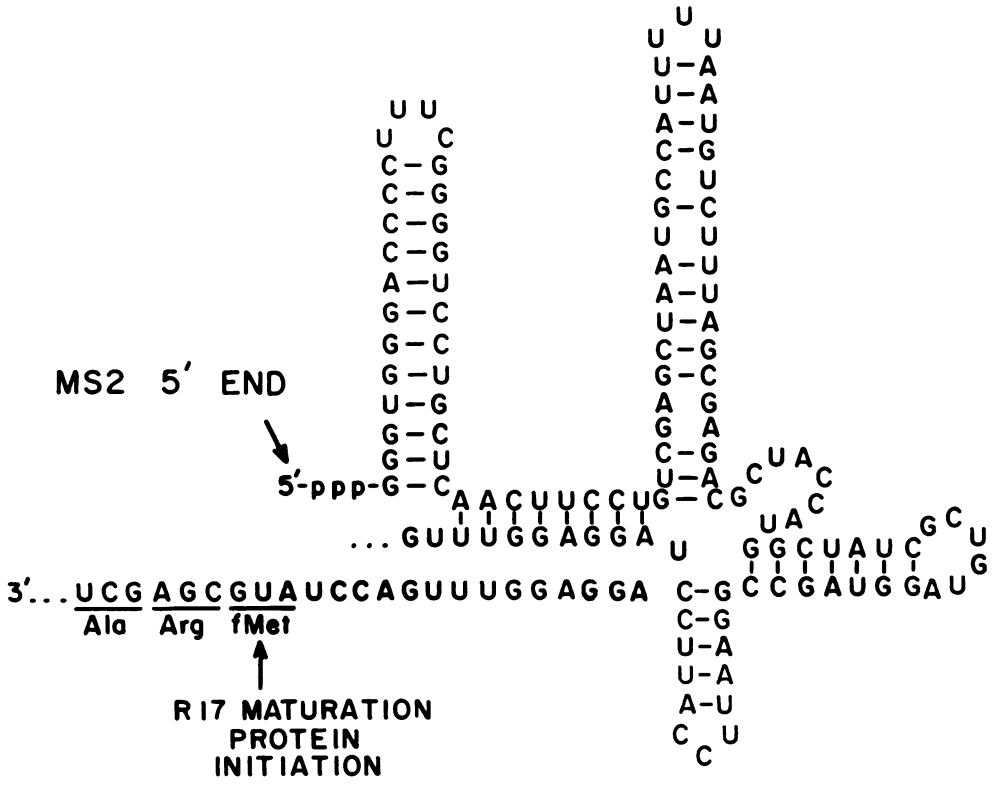

FIG. 1. *Primary and hypothetical secondary structure of the 5′ terminal fragment of MS2 RNA (28) which overlaps, as shown, with the ribosomal binding site for the R17 maturation protein cistron (174).*

sorption, viral RNA penetrates into the cell, a temperature-dependent process requiring specific divalent cations (139) and cellular energy (27). During the penetration step, maturation protein dissociates from the phage particle and enters the bacterial cell in association with the infecting RNA (96, 140). The empty particle, consisting only of coat protein, then desorbs from the pilus into the medium (96, 141, 164).

Within 10 min after entry of the infecting RNA molecule, a new RNA polymerase activity is detectable in the cells (5, 111). This enzymatic system, called RNA synthetase or replicase or polymerase, consists, in the case of $Q\beta$, of a phage-specified polypeptide and three preexisting host polypeptides (85, 93, 162, 195; for a recent review of the properties of this enzyme system, see reference 4). With the infecting RNA molecule (designated a plus strand) as template, the enzyme catalyzes formation of a complementary minus strand which in turn is the template upon which progeny plus strands are polymerized. These progeny molecules are associated with the template in a complex structure designated the replicative intermediate (RI). The RI isolated from cells by phenol extraction has been shown to consist of (i) an intact minus strand, hydrogen-bonded to (ii) one (50) or more (39) incomplete growing plus strand(s), and (iii) an intact plus strand, the 3′ end of which is still associated with the minus strand, whereas the 5′ end has been displaced by the nascent plus strand, forming a single-stranded tail projecting from the duplex (Fig. 3). Evidence for the structure and function of RI has been summarized in detail elsewhere (102, 219). The pertinent observations for the translational process are that molecules of RI are present in the polysome fraction of infected cells (46, 71), and their nascent strands are presumably translated even before the progeny RNA mole-

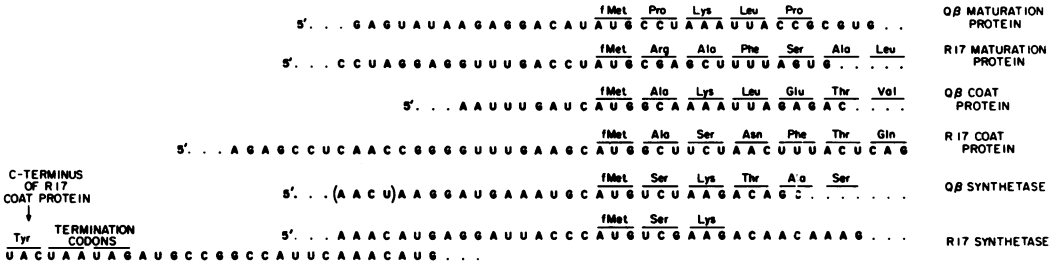

FIG. 2. *Nucleotide sequences of ribosomal binding sites shown with the N-terminal amino acid sequences of the corresponding phage proteins. Data are from the following references: amino acid sequence for R17 coat protein (211), R17 maturation protein (Weiner and Weber, personal communication), R17 synthetase (135), Qβ coat protein (94, 135, 217), Qβ maturation protein (217), and Qβ synthetase (166, 217); nucleotide sequences for the three R17 cistrons (174), Qβ coat cistron (64), Qβ maturation protein cistron (171), and Qβ synthetase (170). The ribosomal binding sequence shown for the R17 coat cistron has been extended six nucleotide residues on the 5' side (81). The sequence of the ribosomal binding site for the f2 coat cistron has also been determined (53); it differs from R17 by only one nucleotide residue in the region preceding the initial AUG triplet. A portion of the fragment from the end of the R17 coat cistron, which overlaps with the synthetase ribosomal binding site, is also shown (128).*

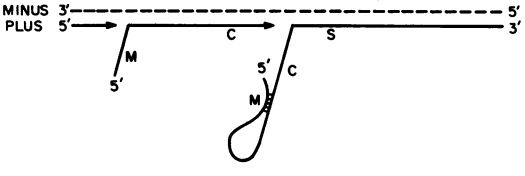

FIG. 3. *Replicative intermediate. Arrowheads indicate the growing points of nascent plus strands. M, C, and S designate ribosomal attachment sites for the maturation protein, coat protein, and synthetase cistrons, respectively. The secondary structure of nascent chains is hypothetical.*

cules are released from the replicating structure. In addition, completed progeny molecules are also found associated with ribosomes (46). Thus, during the replication cycle there are at least three potentially different forms of viral messenger RNA: (i) the parental RNA molecule, (ii) nascent plus strands still partially associated with the replicative intermediate, and (iii) free single-stranded progeny molecules. Minus strands do not appear to function as messenger (159), and there is no evidence for single-stranded fragments of phage RNA in infected cells.

Following the synthesis of progeny RNA molecules and structural proteins (described in detail below), infectious virus appears. Little is known about the assembly of phage particles (for a recent review, see reference 67). In vitro assembly of infectious phage requires RNA, coat protein, and maturation protein (76, 149), and in infected cells there is evidence that interaction between maturation protein and viral RNA precedes the formation of phage particles (25, 84, 147).

PHAGE GENES AND THEIR PRODUCTS

Identification of Phage Genes

Conditional mutants of the RNA coliphages have been used to identify three phage cistrons by complementation tests (55, 69, 194, 196) and by direct analysis of phage proteins. Mutations in each gene lead to characteristic changes in phage development and growth of infected cells, allowing rapid preliminary classification of new mutants (55, 68, 69, 127, 194, 221). Mutants of one group, later identified as RNA synthetase mutants, produce no virus particles, RNA, or enzymatically detectable synthetase (55, 69) and have no effect on the growth of host bacteria. Mutants of the second group, later identified as maturation protein mutants, produce defective, nonadsorbing, ribonuclease-sensitive particles (3, 61, 112, 197); in the case of f2 and related coliphages, the host cell is lysed, whereas maturation protein mutants of Qβ do not lyse the host (69). Mutants of the third group, later identified as coat protein mutants, typically produce normal or greater than normal amounts of viral RNA (68) and RNA synthetase (111) but make no phage particles. They inhibit growth of the host cell without causing lysis.

In su⁻ *E. coli* pretreated with actinomycin or rifampin to inhibit host RNA and protein synthesis, amber mutants of the RNA phages have been used to identify the gene products directly (70, 127, 198, 199). As seen in the up-

permost profile in Fig. 4, infection by wild-type phage produces three electrophoretically separable proteins. One of these (protein 3) corresponds to the major structural protein of the virus (coat protein) and is absent in cells infected by the third group of mutants described above (Fig. 4b). A second (protein 2) corresponds to the minor structural protein of the virus (maturation protein) and is absent in cells infected by the second group of mutants (Fig. 4c). It is also absent in the defective particles which are formed in su cells infected with such mutants (Fig. 4g). The third phage protein (protein 1) has been shown, by in vitro suppression in cell extracts programmed with RNA from group 1 mutants, to be the product of the RNA synthetase gene (121, 127).

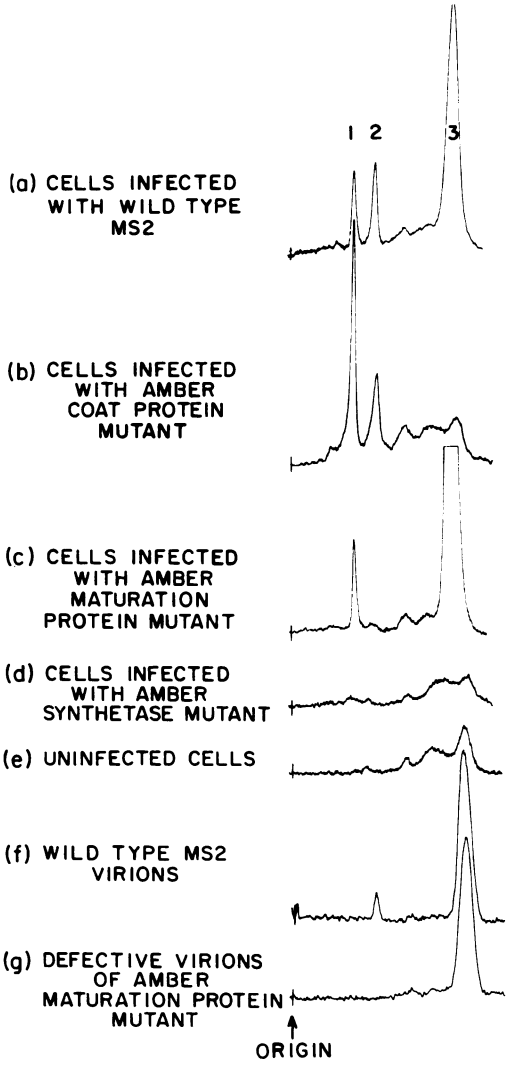

FIG. 4. *Polyacrylamide gel electrophoresis and radioautography of phage proteins in virus particles and in actinomycin-treated E. coli infected with wild type or mutant phage (127). Proteins synthesized from 20 to 40 min after infection were labeled with a mixture of ^{14}C-amino acids. Microdensitometer tracings of the radioautograms are shown. 1, Synthetase; 2, maturation protein; 3, coat protein.*

An additional virus-specified protein has been detected in cells infected with Qβ and in Qβ particles (43, 70, 177). Recently, this fourth protein (designated A_1 or IIb) has been shown to contain the amino acid sequence of the coat protein plus about 200 additional amino acids (70, 122, 217). Thus it appears to result from occasional failure to terminate translation of the coat protein cistron at the usual site and is not the product of a fourth gene. The interesting possibility that this "read-through" polypeptide might have an essential function different from that of normally terminated coat protein has not yet been tested. The production of two functionally different polypeptides from a single cistron due to ambiguity in reading the termination codon would be an extreme example of genetic economy.

Gene Order

Since recombinational analysis has not been possible with the RNA phages, establishing the order of the genes in the RNA molecule required a biochemical approach. Several lines of evidence are now consistent with the 5′ to 3′ (left to right) order: maturation protein-coat protein-synthetase. By use of specific RNA fragments as messenger for in vitro protein synthesis (45, 95, 104, 163, 168), it was tentatively shown that the synthetase cistron is within the 3′ 60% of the RNA molecule, and the coat gene is nearer the 5′ end. Subsequent identification of the synthetase ribosomal binding sequence (*see below*) within the 3′ 60% fragment of the RNA confirmed the location of the synthetase cistron (82). By the same technique, the initiation sites for maturation and coat proteins were shown to be within the 5′ 40% RNA fragment, whereas oligonucleotides from the interior of the coat protein gene were recovered from the 3′ fragment (82), placing the coat gene near the center of the RNA molecule, with the maturation cistron to the left and the synthetase gene to the right. Nucleotide sequence analysis of a small RNA fragment which contains the codons for the C-terminal amino acids of coat protein and

which overlaps with a second fragment containing codons for the *N*-terminal amino acids of synthetase confirmed that these two cistrons are adjacent (128). In a different approach, a collection of Qβ RNA molecules, synthesized in vitro, were labeled to different extents from the 5′ end with ^{32}P, and the amount of ^{32}P present in the ribosomal binding site of the coat cistron was then determined with each population of RNA. It was concluded that the coat protein initiation site is located about 40% from the 5′ terminus (65). Finally, nucleotide sequence determinations have established that the first functional AUG initiation codon, located 130 nucleotide residues from the 5′ end of MS2 RNA (28), is that preceding the codons for the *N*-terminal amino acids of maturation protein. Similar sequence studies with Qβ RNA indicate that an AUG codon, located 62 residues from the 5′ terminus, is the site of initiation of maturation protein in the genome of that phage (171). Thus, after several false leads, the order 5′-maturation protein-coat protein-synthetase-3′ is firmly established.

Gene Products

Each of the three phage proteins has been purified and partially characterized. The coat protein of f2 has a molecular weight of about 14,000. Its amino acid sequence (212) differs from that of R17 (211) and MS2 (100) by only one amino acid substitution. Sequences have also been determined for fr (220) and Qβ (94) coat proteins, which differ more extensively from f2. The coat protein of viruses of the f2 group contains all of the common amino acids except histidine; Qβ coat protein lacks histidine, tryptophan, and methionine (76, 94, 138).

Maturation protein has a molecular weight of about 40,000 (70, 127, 172); its *N*-terminal amino acid sequence has recently been determined (Fig. 2). The phage-encoded component of RNA synthetase has a molecular weight of about 65,000 (85, 93, 127). In the case of Qβ, the amino terminal sequence of this polypeptide has been established (Fig. 2). Although direct sequence analysis has not been done with purified RNA synthetase from the f2 group of phages, it is known from studies on its synthesis in vitro that the *N*-terminal amino acid sequence of the nascent polypeptide is fMet-Ser-Lys (135).

The three phage-encoded polypeptides have an aggregate mass of about 120,000 daltons, thus accounting for over 95% of the estimated number of nucleotides in viral RNA as codons for amino acids.

PHAGE PROTEIN SYNTHESIS IN INFECTED CELLS

Early studies on the synthesis of phage proteins in infected *E. coli* depended on the measurement of RNA synthetase activity in cell extracts or determination of structural antigen, i.e., protein which reacts with neutralizing antisera. Since the RNA synthetase assay depends on both phage RNA template and enzyme present in the extract and, as is now known, host proteins are involved in the enzymatic reaction, it is not clear that the reaction rate was proportional to the amount of phage-specified synthetase.

Subsequently, direct measurement of the rate of synthesis of each phage protein has been carried out in cells pretreated with antibiotic inhibitors of deoxyribonucleic acid-dependent transcription. In infected *E. coli* which have been pretreated with actinomycin or rifampin, radioactive amino acids are incorporated almost exclusively into phage proteins, which can be separated by gel electrophoresis and individually measured, as noted earlier (Fig. 4). Addition of either of these antibiotics prior to or during the early minutes of infection inhibits phage development (41, 59, 60, 117, 133), and therefore phage protein synthesis may not be the same as in a normal infection. However, even in the absence of transcriptional inhibitors, phage proteins are detectable (but not quantifiable), and the overall pattern of phage protein synthesis appears similar to that seen in infected cells treated with the antibiotics (142, 187). In some instances, rifampin seems to inhibit only the terminal events of phage assembly or release, or both (33, 34, 142).

Infection with Wild-Type Phage

Figure 5 shows the kinetics of synthesis of phage proteins and RNA in infected *E. coli* pretreated with actinomycin compared with the kinetics of infectious phage production. As seen in the figure, RNA synthetase is the first detectable phage protein, appearing between 5 and 15 min after infection. Its rate of synthesis increases until about 25 min after infection and then rapidly diminishes. In actinomycin-treated cells, a low rate of synthetase formation continues, whereas in rifampin-treated cells no synthetase formation is detectable after 20 to 25 min (41, 181). Enzymatic analysis of synthetase production in infected cells reveals similar kinetics (69, 111).

Maturation protein synthesis begins later than that of synthetase, increases in rate, and

continues later in the infectious cycle at a reduced rate both in actinomycin-treated and rifampin-treated cells (41, 181, and Fig. 5). Although coat protein synthesis is difficult to detect early in infection due to the residual background of host protein synthesis, it appears that its production begins later than that of synthetase (*see below*). Coat protein formation then rapidly increases in rate and, in contrast with the other two phage proteins, it continues to be made at a near maximal rate throughout most of the phage growth cycle. The approximate molar ratios of RNA synthetase-maturation protein-coat protein made during the developmental cycle in actinomycin-treated cells are 1:2:20 (127, 198).

It is interesting to note by way of contrast that, in cells infected with poliovirus, an animal virus similar in structure to the RNA coliphages, all viral proteins appear to be made in equimolar amounts due to translation of the mRNA as a unit (80, 156). Clearly, in the development of the RNA coliphages, special mechanisms are operating which determine the amount of each phage protein made and its time of synthesis, in accord with the role or roles each plays in the infectious process.

Infection with Phage Mutants

A clue to the possible regulatory role of a given phage protein can be obtained by observing the pattern of phage protein synthesis in the absence of functional molecules of that particular protein. This has been done by infecting actinomycin-treated su⁻ *E. coli* with amber mutants, or by infecting at a nonpermissive temperature with temperature sensitive (*ts*) mutants. The results of these experiments can be summarized as follows. (i) With

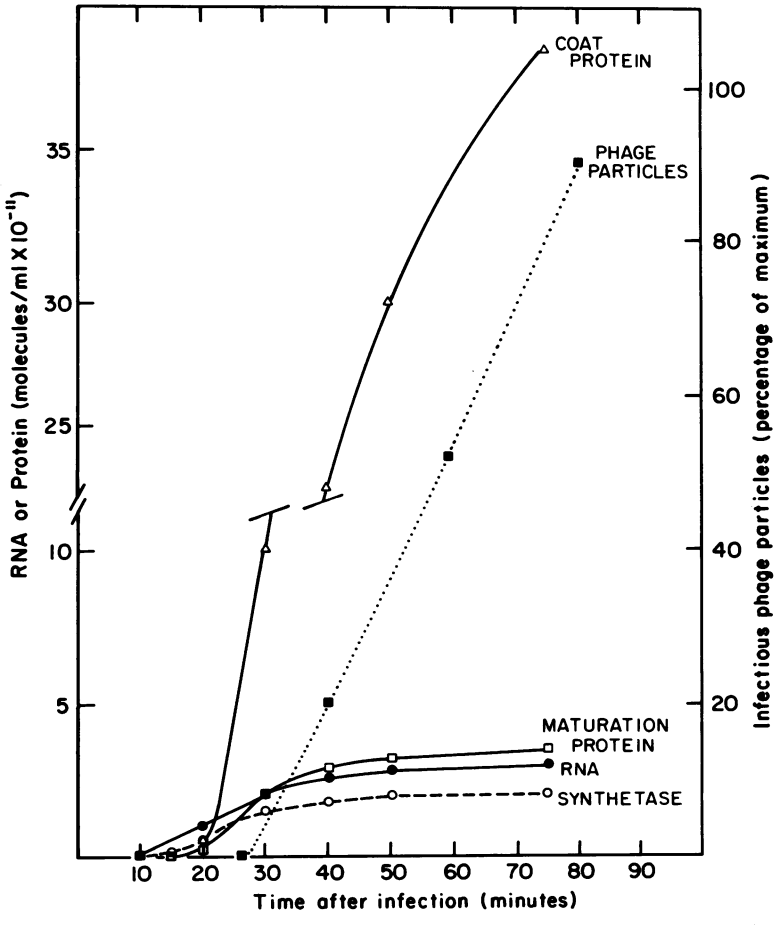

FIG. 5. *Kinetics of synthesis of phage proteins, RNA, and infectious virus particles in actinomycin-treated cells (127, 133).*

amber synthetase mutants, no phage proteins are detectable (Fig. 4d) and progeny viral RNA is not formed. With *ts* synthetase mutants, the early synthetase protein is formed normally but neither of the other phage proteins is detectable (Fig. 6). Thus, on the infecting RNA template, only the synthetase gene appears to be translated. (ii) When amber maturation protein mutants are used, the synthesis of coat protein and synthetase are essentially the same as after infection with wild-type phage (Fig. 4c). By use of an amber mutant which produces a measurable fragment of maturation protein, it has also been found that the amount of this fragment is about equivalent to the amount of maturation protein produced normally; i.e., the absence of functional maturation protein has not influenced translation of the maturation cistron (127). (iii) With amber coat protein mutants, two different patterns have been observed, depending on the site of the mutation. Some mutants (nonpolar mutants) lead to excessive formation of synthetase (Fig. 4b), due to continued production late in the infectious cycle, and to a less marked increase in maturation protein synthesis (127, 198). This elevated synthetase production in cells infected with certain coat protein mutants has also been demonstrated enzymatically (55, 69, 111, 115). Other mutants of the coat protein gene (polar mutants) present the same phenotype as synthetase mutants; i.e., they produce little or no synthetase, detectable by enzymatic analysis (55, 115), conversion of parental RNA to the double-stranded replicative form (103, 148), or polyacrylamide gel electrophoresis of infected-cell extracts (Kozak and Nathans, *unpublished data*). Hence little phage RNA or protein of any type is made. The latter polar mutant type (e.g., *sus3* of coliphage f2) contains a nonsense mutation at a site corresponding to the sixth amino acid residue from the *N*-terminus of the coat protein, whereas the former nonpolar mutants contain a mutation at a site corresponding to amino acid residue 50, 54, or 70 (115, 194). Unexpectedly, amber coat protein mutants also appear to produce little or no coat protein fragment in infected cells, which has led to the suggestion that functional coat protein may be required for efficient translation of the coat cistron (188). However, with a *ts* coat protein mutant at nonpermissive temperatures, coat protein is made in normal amounts (D. Nathans, *unpublished data*).

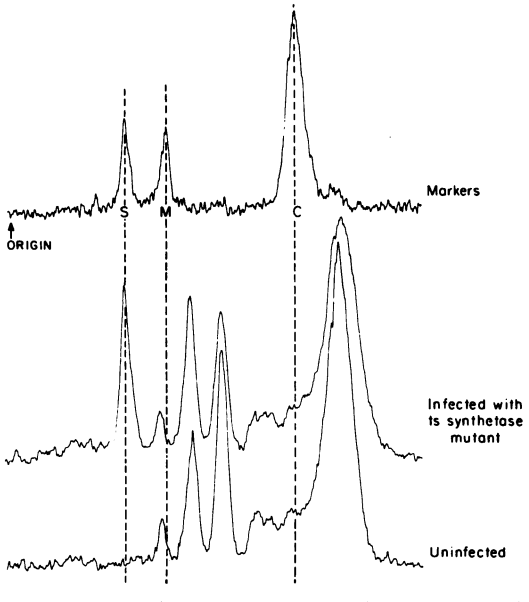

FIG. 6. *Phage proteins translated from parental RNA. A log-phase culture of E. coli C3000-38 (uridine⁻) was pretreated with actinomycin (15 μg/ml) for 7 min and then infected at 43 C with a ts synthetase mutant of MS2. Uridine was withheld from the infected bacteria. Proteins synthesized from 4 to 15 min after infection were labeled with ^{14}C-valine and isoleucine and were analyzed by polyacrylamide gel electrophoresis and radioautography as described previously (127). Comparison of the proteins made in infected cells with the residual protein synthesis in similarly treated, uninfected cells reveals a single phage-specific peak, which has the mobility of synthetase. Top profile shows phage proteins synthesized late in cells infected with wild-type MS2: S, synthetase; M, maturation protein; C, coat protein.*

The studies on phage protein synthesis in cells infected with wild-type or mutant phage thus indicate that the synthesis of each phage protein is regulated. (i) Translation of the infecting RNA results in the formation of synthetase but not of complete coat protein or maturation protein. (ii) The position-dependent polar effect of coat protein nonsense mutations suggests that translation of the synthetase gene requires prior translation of the first part of the coat protein gene. (iii) The formation of synthetase ceases soon after infection, provided that functional coat protein is produced. (iv) Maturation protein is synthesized at a low rate compared to coat protein during the late stages of the infection. The possible mechanisms underlying each of these instances of differential translation of the polygenic phage RNA have been investigated in an in vitro protein-synthesizing system programmed with phage RNA, which we consider below.

PHAGE PROTEIN SYNTHESIS IN E. COLI EXTRACTS

Protein Products

Under appropriate conditions, complete molecules of all three phage proteins can be synthesized by *E. coli* extracts programmed with RNA isolated from the phage. Coat protein was first identified as the major product by correspondence of the tryptic peptides of the product with the tryptic peptides of coat protein prepared from phage particles (13, 125, 126). Subsequently it was shown that the synthetic product and authentic coat protein cochromatograph on guanidine-Sephadex columns (125), and co-electrophorese in sodium dodecyl sulfate-acrylamide gels (30, 83, 185), indicating that the molecular weight of the synthetic product is similar to that of the reference coat protein. Moreover, by using RNA from coat protein nonsense mutants as messenger, *N*-terminal peptides of the coat protein are made and terminated prematurely at the site of the nonsense mutation (213); complete molecules of coat protein are produced only when tRNA from su^+ bacteria is added to the system (13, 16).

In vitro formation of synthetase has also been clearly demonstrated. The synthetic product contains histidine which is absent from coat protein, and co-electrophoreses with synthetase extracted from infected cells (30, 83, 185, 200). With mRNA from synthetase nonsense mutants, production of the putative synthetase peak is specifically stimulated by addition of su^+ tRNA (13, 121, 127), confirming the identification of the product.

The presence of maturation protein among the in vitro products was more difficult to prove. This was due to the fact that very little and perhaps no maturation protein is made when intact phage RNA is used as messenger (*see below*). Furthermore, an *N*-terminal fragment of synthetase appears to accumulate in the extracts and migrates on gel electrophoresis at about the same rate as maturation protein (114, 121). However, by using mRNA from a polar coat protein mutant and by further inhibiting production of synthetase with exogenous coat protein (*see below*), it has been clearly demonstrated by polyacrylamide gel electrophoresis that whole molecules of maturation protein are made (114). Furthermore, the ^{35}S-methionine-labeled tryptic peptides of the synthetic product co-electrophorese with those from maturation protein isolated from phage particles (114). When RNA with an amber mutation in the maturation protein cistron is used as messenger, one of the tryptic peptides is absent from the product as expected (114).

The "read-through" polypeptide observed in cells infected with coliphage Qβ is also synthesized in cell-free extracts programmed with Qβ RNA (83).

Nucleotide sequence analysis of the intercistronic region between the R17 coat and synthetase genes revealed a potential initiation codon (AUG) followed six triplets later by the termination codon UGA (128 and Fig. 2). Thus, an additional product—the hexapeptide fMet-Pro-Ala-Ileu-Glu-Thr—might be translated from the R17 genome. Attempts to detect formation of the hypothetical hexapeptide, however, have been unsuccessful (18). There is no potential initiation codon in the intercistronic region in MS2 RNA (118).

It has been apparent from the earliest experiments on phage RNA-directed protein synthesis in vitro that coat protein is the predominant product (126). For example, with f2 RNA as messenger, the ratio of coat-synthetase-maturation protein formed was 1:0.3:0.06 (104). In other experiments, no maturation protein was detectable (83, 135, 185). Thus, even in cell-free extracts there is differential translation of the three phage genes. As will be discussed later, this manifestation of translational regulation reflects differential rates of initiation of the three phage proteins.

Initiation of Phage Proteins

The discovery that a specific initiator tRNA, fMet-$tRNA_f$, is involved in bacterial protein synthesis was first made with coliphage RNA as messenger (1, 213). Subsequently, all three phage proteins were shown to be initiated by fMet (104, 114, 200), and much of the work done on the mechanism of initiation has been carried out with phage RNA. Although it is not our intention to review the general aspects of this work in detail, we need to summarize certain results for later reference.

Formation of the initiation complex with coliphage mRNA and fMet-$tRNA_f$ requires 30*S* ribosomal subunits, guanosine triphosphate (GTP) and initiation factors (22). Only after formation of the 30*S*-containing complex is a 50*S* subunit added to yield a 70*S* ribosomal complex (51, 56, 131, 132). The initiation factor preparation has been fractionated into three proteins, designated IF1, IF2, and IF3 (also called F_1, F_2, and F_3; A, C, and B; or FI, FIII, and FII, respectively). Of particular importance for later discussion is the observation that whereas IF2 is sufficient for ribosome

binding to synthetic polynucleotide templates containing an AUG initiator codon (146), for relatively labile, nonspecific binding to T4 mRNA (145, 146) or for binding to various sites on formaldehyde-denatured MS2 RNA (7), addition of IF3 is required for stable binding of 70S ribosomes to the specific initiation sites on natural mRNA species including R17 (205), MS2 (7, 157), T4 mRNA (145), and λ mRNA (6).

Specific assays for the initiation of each of the three phage proteins have been developed. The simplest assay determines the amount of each initial dipeptide formed under conditions where translocation does not occur due to depletion (106) or inhibition (154) of elongation factor G. Since the *N*-terminal amino acid of each phage protein is different (Fig. 2), the initial dipeptides are distinctive: fMet-Ala for coat protein (1, 213), fMet-Ser for synthetase (104, 174), and fMet-Arg for maturation protein in the case of f2 and R17 (106, 174); the initial dipeptide of Qβ maturation protein is fMet-Pro (217). These dipeptides have been measured by determining the amount of amino acid-labeled Ala-, Ser-, or Arg-tRNA bound in the initiation complex by membrane filtration (202). However, background levels in the absence of phage RNA are often high, especially with Arg-tRNA. A more satisfactory assay is the measurement of the fMet-labeled dipeptides isolated by paper electrophoresis after hydrolysis of the dipeptidyl-tRNA species (106, 154, and Fig. 7). Another initiation assay depends on the ability of ribosomes in an initiation complex to protect against ribonuclease digestion that region of the phage RNA which is bound to the ribosome. After digestion of the bulk of the RNA molecule, which is not directly bound, the residual protected oligonucleotides have been isolated and their nucleotide sequences have been determined (53, 64, 170, 171, 174). By matching the nucleotide sequences with the *N*-terminal sequences of phage proteins, each oligonucleotide has been identified as the initiation site for a specific phage protein (Fig. 2). Although the yields of individual fragments have been taken as a measure of relative initiation frequencies, it has not been shown that the three initiation complexes are equally stable during isolation on sucrose gradients or during ribonuclease digestion.

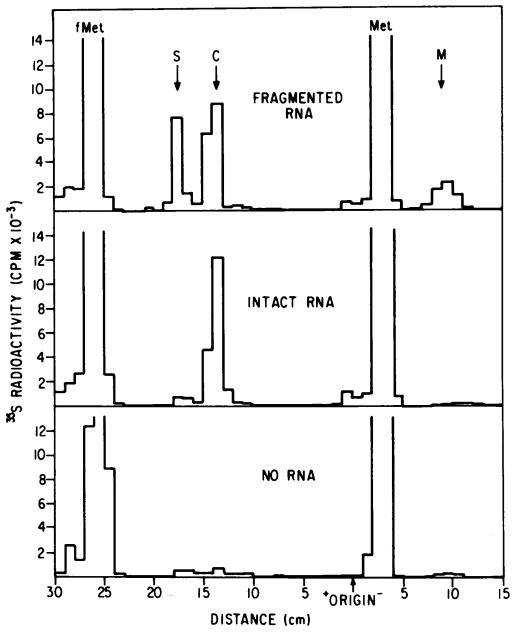

FIG. 7. *Initiation of phage proteins: electrophoretic separation of initial ^{35}S-methionine-labeled dipeptides formed with intact MS2 RNA or with MS2 RNA which had been fragmented by heating for 8 min at 70 C in 0.01 M Tris-hydrochloride, 0.01 M magnesium acetate at pH 7.5 followed by dialysis against EDTA. Reaction conditions and electrophoretic analyses were similar to those described in references 106 and 154. S: initial synthetase dipeptide, fMet-Ser; C: initial coat dipeptide, fMet-Ala; M: initial maturation protein dipeptide, fMet-Arg.*

Elongation and Termination of Phage Proteins

The availability of a messenger with much of its nucleotide sequence known and an in vitro product, the amino acid sequence of which is also known, has permitted rather precise experiments on the elongation and termination steps of protein synthesis. Although there is no evidence for unusual mechanisms in elongation and termination of phage proteins, we shall nonetheless summarize recent experiments which appear to have general significance.

After formation of the 70S initiation complex between ribosomal subunits, phage mRNA, and fMet-tRNA$_f$, addition of elongation factors Tu and Ts in the presence of GTP allows Ala-tRNA, specified by the second codon of the coat cistron, to bind to the complex, and the initial dipeptidyl-tRNA is formed. Whether additional aminoacyl-tRNA species can bind to the complex prior to translocation is not entirely clear. In one series of experiments, the Ser-tRNA corresponding to the third codon of the f2 coat cistron (or Lys-tRNA in the case of Qβ) was unable to bind to the ribosome in the absence of functional G factor (153, 155), whereas other experiments

suggest that the aminoacyl-tRNA species corresponding to the third and fourth codons of the coat cistron can bind prior to translocation (188a). In any case, subsequent addition of G factor and GTP changes the position of the ribosome on the mRNA as determined by nucleotide sequence analysis of the messenger fragment remaining after ribonuclease digestion of the unprotected regions. Prior to translocation, the 3′ terminus of the protected f2 RNA fragment is UUU; after translocation, the 3′ terminus is UUUACU [i.e., the ribosome has advanced three nucleotides further towards the 3′ end of the messenger (54)]. When the pre-translocation complex is treated with ribonuclease and then the protected messenger fragment is translated, the product is primarily a pentapeptide derived from the *N*-terminal end of f2 coat protein: fMet-Ala-Ser-Asn-Phe. Translation of the messenger fragment protected within the post-translocation complex, however, produces primarily the *N*-terminal hexapeptide of f2 coat protein: fMet-Ala-Ser-Asn-Phe-Thr (54). Thus progression of the ribosome, triplet by triplet, has been directly demonstrated with phage RNA as it has with synthetic oligonucleotides (190).

The rate of polypeptide elongation has also been estimated. By correlating the position of amber mutations in the coat cistron with the rate of release of ribosomes from phage mRNA in a synchronized reaction, it has been found that the rate of elongation of coat protein in vitro is about 25 to 30 amino acids per min (216). This average rate has been confirmed by other techniques (176). However, there is some indication that the rate of translation may vary for different regions of the coat cistron (72).

In regard to termination of phage proteins, both natural termination at the end of cistrons and termination at internal nonsense codons, produced by mutation, have been studied. In either case, ribosomes dissociate from the messenger and a nascent polypeptide is released, free of tRNA, when a nonsense codon is encountered (11, 214, 216). The tandem nonsense triplets (UAA-UAG) involved in terminating the R17 coat protein cistron have been directly identified by nucleotide sequence analysis of a fragment of R17 RNA (128 and Fig. 2). In contrast, the Qβ coat protein termination signal has been deduced indirectly to be a single UGA triplet (217). Since UGA can be translated to a limited extent even in su$^-$ bacteria (121), it is an inefficient termination signal. This probably accounts for the presence in Qβ-infected cells of small amounts of a fourth polypeptide resulting from progression of some ribosomes through the coat cistron termination signal, as mentioned previously.

In addition to an appropriate codon, polypeptide termination in vitro requires certain soluble proteins, but there is no evidence for participation of a tRNA which recognizes the termination codon (15). The role of the soluble proteins or release factors was first discovered in studies on the release of the *N*-terminal hexapaptide of nascent coat protein by using RNA from phage with an amber codon in the next position (15). By employing the initiator triplet AUG to initiate, and different nonsense triplets to terminate, it was possible to identify two release factors, one (R1) specific for the termination triplets UAA or UAG and a second (R2) specific for UAA or UGA (21, 160). [In addition, a third soluble protein, α or S, stimulates polypeptide release (17, 21).] By using antisera prepared against purified R1 or R2, it has been shown that either factor is sufficient to effect termination and release of the coat protein of coliphage R17 (18). This is consistent with the ability of both R1 and R2 to respond to UAA, the first of the tandem nonsense triplets at the end of the R17 coat cistron. Similar experiments have indicated that termination of coliphage R17 synthetase can also be mediated by either R1 or R2 (18), implying that UAA is the first (or only) termination codon at the end of that gene. Detailed studies on the termination of maturation protein have not yet been reported.

Although a good deal is known about the termination reaction, the picture is not yet complete. Presumably each release factor recognizes particular termination triplets, but how they induce cleavage of the peptidyl-tRNA is not understood. There is evidence (17, 21) that the peptidyl-tRNA is released from the peptidyl or donor site on the ribosome (operationally, the puromycin-sensitive site), and thus the hydrolysis of peptidyl-tRNA may be catalyzed by the ribosomal peptidyl transferase. In support of this hypothesis, recent experiments indicate that 50*S* ribosomal subunits can be manipulated so that peptidyl transferase will hydrolyze ribosome-associated fMet-tRNA in the absence of R factors (20). Furthermore, a number of antibiotics that inhibit peptidyl transferase also inhibit termination (17, 193, 201).

REGULATION OF PHAGE PROTEIN SYNTHESIS IN VITRO

As previously noted, strikingly different amounts of the three phage proteins are syn-

thesized in *E. coli* extracts programmed with phage RNA. Although one could imagine mechanisms of translational control operating at the initiation, elongation, or termination steps of protein synthesis, all present evidence points to the initiation reaction as the controlling step. As discussed below, the initiation reaction may be influenced by dynamic conformational features of the phage RNA, by the nucleotide sequence near the initiation site of each cistron, by the ability of ribosomes or initiation factors, or both, to discriminate among cistrons, and by specific binding of viral proteins to the mRNA.

Differential Initiation as a Function of RNA Conformation

The relative amounts of coat protein, synthetase, and maturation protein initiated in *E. coli* extracts depend on the integrity and conformation of the viral RNA. With "native" RNA, isolated from phage particles by phenol extraction and shown to be intact by sedimentation through sucrose gradients containing formaldehyde, only coat protein is initiated, as shown by the initial dipeptide assay (Fig. 7) and by nucleotide sequence analysis of f2 or Qβ RNA fragments recovered from the 70*S* initiation complex after ribonuclease digestion (53, 64). Also consistent with the observed exclusive initiation of the coat protein gene is the finding that only one ribosome can attach to phage RNA under conditions in which polypeptide chain elongation is prevented (36, 79, 92, 189, 215, 216). That the single site available for ribosome binding on native viral RNA is at the beginning of the coat cistron was confirmed by first forming the 70*S* initiation complex and then, in the presence of aurintricarboxylic acid (ATA) to prevent new initiations, adding the components required for polypeptide elongation; a single product identified electrophoretically as coat protein was synthesized (215). Alternatively, if the 70*S* initiation complex is first digested with ribonuclease to remove the unprotected regions of the viral RNA, and the messenger fragment protected by the bound ribosome is then translated, the product is the *N*-terminal pentapeptide of coat protein (54, 98). We can thus designate native viral RNA as $M^-C^+S^-$ to indicate that the coat initiation site (C) is open, whereas the maturation protein (M) and synthetase (S) sites are not available for translation.

Exclusive initiation of coat protein stringently depends on the intactness of the messenger, which can only be demonstrated in the presence of denaturing agents; this probably explains the discrepant results on relative initiation of phage proteins reported by different investigators (106, 113, 153, 155, 166, 203). Intentional fragmentation of the RNA by limited exposure to ribonuclease (105, 106), elevated *p*H (170, 202), autoradiolysis (174), or heating under appropriate conditions (Fig. 7) allows all three proteins to be initiated. This is evident from the kinetics of histidine incorporation (105), analysis of the initial dipeptides (106, 152, 202 and Fig. 7), and analysis of the protected oligonucleotide sequences isolated after ribonuclease digestion of the 70*S* initiation complexes formed with fragmented viral RNA (170, 174). Thus, fragmented RNA can be designated $M^+C^+S^+$ to indicate that all three initiation sites are open, presumably due to the loss of certain conformational restraints upon limited cleavage of the messenger.

The observed increase in initiation of synthetase and maturation protein as well as a decrease in coat initiation, brought about by heating the RNA in the presence of magnesium, have been attributed by some investigators to conformational changes of the intact RNA (42, 110). Consistent with this interpretation are the slight hyperchromic effect observed under these conditions (110) and particularly the observation that the apparent closing of the C site can be reversed by dialysis against ethylenediaminetetraacetic acid (EDTA) and brief heating at 45 C (42). However, the integrity of the heated RNA was not rigorously demonstrated in these studies. In our hands, similar heat treatment (*see* legend to Fig. 7) resulted in breakage of the RNA, as determined by sedimentation in sucrose gradients containing formaldehyde. With the heat-fragmented RNA as template, the S and M sites were readily initiated, whereas initiation at the C site was depressed, relative to intact MS2 RNA; dialysis of the fragmented RNA against EDTA followed by brief incubation at 45 C restored initiation at the C site. Therefore the reversible decrease in coat initiation appears to involve aggregation of RNA fragments. Thus, fragmentation, rather than a thermal-induced conformational change of the intact RNA molecule, may account for all of the observed template properties of heated phage RNA.

When the conformation of viral RNA is partially disrupted by mild formaldehyde treatment, there is a dramatic increase in initiation of maturation protein and synthetase, assayed both by initial dipeptide formation (109) and

by identification of the protected ribosomal binding sites (7). The similar effects of formaldehyde treatment and fragmentation on ability to initiate the synthetase and maturation protein cistrons strongly suggest that specific conformational features in native RNA restrict initiation at these two sites.

Another line of investigation also has led to the conclusion that the secondary structure of phage RNA limits initiation of the synthetase cistron. It has been known for some time that there is a delay in the appearance of synthetase in vitro compared to the appearance of coat protein (30, 31, 36, 104, 134, 185), and that this reflects a delay in synthetase initiation (52). Related to this lag in synthetase formation is the phenomenon of polarity observed with certain coat protein mutants, such as *sus3*, which has been discussed earlier. As already noted, *sus3* has a nonsense mutation corresponding to the sixth amino acid residue from the *N*-terminus of the coat protein, and cells infected with this mutant show very little formation of RNA synthetase. The polar effect on synthetase formation has also been demonstrated in vitro. In an extract from su^- *E. coli*, much less synthetase is made with *sus3* mRNA compared with wild-type messenger (14, 36, 104, 114, 222); addition of tRNA from su^+ *E. coli* leads to enhanced synthetase formation (36, 105, 222). Since artificial perturbation of the RNA conformation (by formaldehyde, heat, etc., as discussed above) opens the synthetase initiation region, it has been suggested that ribosomes translating the coat protein gene beyond the *sus3* mutation similarly open the S site.

Consistent with this hypothesis is the report that addition of ATA, within 2 min after coat protein synthesis has begun, completely prevents production of synthetase while allowing coat elongation to continue. If ATA is added after 10 min of incubation, however, it inhibits neither synthetase nor coat protein production since both cistrons have been initiated by that time (52). From similar experiments in which ATA was added at various times after initiation of coat translation, it has been estimated that the synthetase cistron becomes available for initiation around the time when elongation of the coat polypeptide reaches the 40th amino acid residue (176).

Recently, determination of the nucleotide sequence of an extensive stretch of the coat protein and adjacent synthetase cistrons has revealed a probable region of hydrogen bonding, 21 nucleotides long, between the synthetase initiation site and codons 24 to 32 of the coat cistron, which could elegantly account for the closed state of the S site (W. Fiers, *personal communication*). Polarity would then be attributed to failure of ribosomes to reach, and thus open, this region of the RNA if a nonsense mutation is present in the preceding portion of the coat cistron.

In contrast with synthetase, initiation of maturation protein is not affected by conformational changes that occur during translation of the coat gene. Thus, amber mutations in the coat cistron have no polar effect on production of maturation protein in vitro (104, 114).

Another important observation which is probably related to control of polypeptide initiation by the conformation of the mRNA concerns the messenger function of replicating phage RNA isolated from infected cells. As indicated earlier, the RI consists of an intact minus-strand template with one or more nascent single-stranded plus chains extending from it (Fig. 3). In vitro, replicative intermediate initiates about five times as much maturation protein, relative to the total protein initiated, as does single-stranded RNA isolated from phage particles (150). Presumably, the conformation of the 5′ end of the single-stranded RNA chains in the RI is different from that of completed RNA molecules, allowing ribosomes access to the M site on the nascent strands. Only a fraction of the maturation protein molecules initiated on RI in vitro are actually completed, however (150), in contrast with coat protein which, therefore, appears as the major product directed by RI (35, 150). This failure to produce complete molecules of maturation protein could be explained by assuming that only the shortest nascent strands have open M sites, and these strands are not long enough to contain the entire maturation protein cistron. According to this notion, as each nascent RNA chain is synthesized in the infected cell, a ribosome attaches to the M site in the short 5′ tail; as the RNA chain is elongated, that ribosome proceeds to translate the maturation protein cistron, but folding of the elongated RNA strand (Fig. 3) prevents additional ribosomes from initiating at the M site. Thus, in infected cells, RI could function as messenger for a limited amount of maturation protein. Consistent with the postulated closing of the M site after the nascent RNA chain has attained a certain length is the observation that ribosomes can bind to the M site (assayed by analysis of the protected oligonucleotide sequence) in enzymatically synthesized 5′ terminal fragments of $Q\beta$ RNA up to about 500 nucleotides in length, whereas in full-length molecules the M site is no longer accessible to ribosomes (171).

Evidence for Specificity of Ribosomes and Initiation Factors

The above observations on translation of fragmented or formaldehyde-treated RNA, RNA from polar phage mutants, and replicating viral RNA clearly indicate that the overall conformation of phage RNA influences initiation of the synthetase and maturation protein genes. Since the ribosome binding sites preceding the initial AUG codon differ in nucleotide sequence for each phage cistron (Fig. 2), an additional level of control may result from the affinity of ribosomes or initiation factors, or both, for each of these sites, even when they are free of interaction with distant parts of the RNA molecule. In this case one could imagine that the same ribosome or initiation factor can recognize all three sites but bind with different affinity to each, or a separate class of ribosomes or initiation factors might recognize the initiation signal of each cistron. At present there is evidence for both ribosome and initiation factor specificity.

The strongest evidence that ribosomes can discriminate among cistrons is the striking observation that, in contrast with *E. coli* ribosomes, *Bacillus stearothermophilus* ribosomes initiate maturation protein, but not coat protein or synthetase (107), as determined both by the initial dipeptide assay (108) and by analysis of the oligonucleotides protected by the bound ribosomes (174). By suitable mixing experiments, the 30*S* subunit has been shown to determine the cistron specificity (108). In contrast with *B. stearothermophilus*, ribosomes from *B. subtilis* show no activity with phage RNA (113).

These findings raise the interesting possibility that within the *E. coli* cell, in which all three phage cistrons are translated, a different class of ribosomes might be responsible for initiating each cistron. As is now well known, 30*S* ribosomal subunits are structurally heterogeneous with respect to certain proteins (204); this might be a reflection of functional heterogeneity. Although no critical test of this notion has yet been made, recent experiments have shown that when MS2-infected *E. coli* are exposed to kasugamycin, an aminoglycoside antibiotic which acts on the 30*S* ribosomal subunit (63, 169) to inhibit initiation (62), synthesis of maturation protein is markedly inhibited compared with coat protein synthesis (Kozak and Nathans, *unpublished data*). Kasugamycin has a similar differential effect on formation of the initial phage dipeptides in vitro. These observations suggest either that ribosomes of different kasugamycin sensitivity initiate translation of the maturation and coat protein cistrons, or that the antibiotic differentially affects the ability of a single population of ribosomes to bind to the two initiation sites on the phage RNA. More direct experiments in which ribosomes which have initiated a single phage protein are reisolated and tested for their ability to initiate the other phage proteins might be most informative.

In regard to cistron specificity of initiation factors, two lines of evidence point to the tentative conclusion that multiple species of IF3, with cistron-selective activities, are present in *E. coli*. The first line of evidence concerns the altered initiation of R17 or MS2 proteins by initiation factors from T4-infected cells, compared to factors from uninfected cells. Analysis of the oligonucleotide sequences protected by bound ribosomes from T4-infected or uninfected cells, with initiation factors from T4-infected cells, has revealed that appreciable binding occurs only at the beginning of the maturation protein cistron (175). The observed failure to stimulate coat protein initiation could explain why, in cell-free extracts, initiation factors from T4-infected cells fail to promote MS2- or R17-directed protein synthesis, although the extracts are active with T4 or T5 mRNA (29, 87, 158). The deficiency in the T4 initiation factor preparations has been specifically identified as IF3 (99a, 143). This group of experiments suggests that there is selective inactivation of coat and synthetase-specific initiation factor(s) following T4 infection and therefore that uninfected *E. coli* may contain components of IF3 which discriminate between these cistrons and that of the maturation protein.

The second line of evidence concerns the fractionation of IF3 activity from uninfected *E. coli* into components which show messenger or cistron specificity. By assaying IF3 activity with late T4 mRNA (measuring amino acid incorporation or lysozyme synthesis) or with MS2 RNA, it was found that the ratio of activities with the two messengers varied markedly in different purified-factor fractions (99a, 144). Moreover, various fractions of IF3 differed in their ability to translate MS2 coat or noncoat proteins, measured as valine *versus* histidine incorporation (144). In more detailed experiments, a purified subfraction of IF3, known to be more active with MS2 RNA than with T4 mRNA, was tested by the ribosome-binding assay for its effect on initiation of each cistron

in formaldehyde-denatured MS2 RNA (7). In the absence of IF3, the proportions of C-S-M sites isolated were 1:2.2:1; in the presence of the subfraction of IF3, the proportions were 2.5:2:1. Since the total binding of MS2 RNA to ribosomes was about the same in the two instances, this result was interpreted to indicate that the particular subfraction of IF3 used preferentially stimulates ribosomal binding to the coat protein initiation site (7). The identification of other subfractions of IF3, specific for initiation of the synthetase and maturation protein cistrons, has not yet been reported.

In addition to possible cistron-specific IF3 fractions, an inhibitory protein (called factor i) has been isolated recently from *E. coli* ribosomes which has been shown to interact with IF3 and specifically reduce initiation at the C site of MS2 RNA (50a). The i protein also binds to MS2 RNA (50a), and hence its precise mode of action is not clear at the present time.

To understand how a particular subfraction of IF3 might selectively promote ribosomal binding at one or another cistron, we need to know what structural feature in natural mRNA constitutes the signal for ribosomal attachment. The simplest hypothesis is that the codon AUG (or GUG) is the complete initiation signal, and that ribosomes bind wherever this codon occurs on the messenger. The alternative to this "minimal recognition" notion is that the primary or secondary structure, or both, around certain AUG codons comprises a necessary part of the initiation signal. Several experiments have been reported bearing on these hypotheses.

Of the many AUG and GUG codons present in phage RNA, only one—at the beginning of the coat protein cistron—actually serves as an initiation site in native RNA. The simplest explanation for this limited initiation, consistent with the minimal recognition hypothesis, is that the extensive hydrogen-bonded regions in viral RNA conceal all the other AUG and GUG codons, preventing ribosome attachment. This interpretation is supported by the observation that partial elimination of the secondary structure by mild formaldehyde treatment markedly increases the number of sites to which ribosomes can attach. Initiation occurs on formaldehyde-treated RNA not only at the M, C, and S sites but also at several other internal AUG or GUG codons, as revealed both by initial dipeptide formation (109) and by isolation of the oligonucleotides protected from ribonuclease by the bound ribosomes (7). However, in addition to unfolding the viral messensomal recognition sequences may be created by this means. Indeed, when the secondary structure of viral RNA is disrupted by limited fragmentation, by using techniques that do not ger, formaldehyde chemically alters individual base residues in the RNA, and thus new ribo- involve chemical modification of bases, adventitious initiations are not observed; ribosomes bind only at the beginning of the phage cistrons (170, 171, 174 and Fig. 7) and not at other AUG and GUG codons which are probably exposed in the fragmented messenger. Ribosomal binding at only the three "correct" AUG codons in fragmented RNA suggests that the nucleotide sequence preceding or following each of these triplets serves as part of a specific signal for initiation.

An important series of template competition experiments has confirmed that the initiation signal in natural mRNA is not merely the codon AUG. In the presence of IF2 and IF3, ribosomes bind to T4 mRNA in preference to AUG triplets or various synthetic polynucleotides (145). This preferential binding to and translation of the natural messenger have been demonstrated by enzymatic analysis of T4 mRNA-directed lysozyme synthesis, and by observation in the electron microscope. IF3 is responsible for this selective binding to the natural messenger; in the presence of only IF2, AUG triplets and synthetic oligonucleotides compete very effectively with T4 mRNA for ribosome attachment (145). These observations are similar to the specific stimulation of ribosomal attachment at the C site with formaldehyde-treated MS2 RNA upon addition of a subfraction of IF3 (7). In this case, IF3 has presumably stabilized binding at the C site at the expense of binding at other sites.

It is thus fairly clear that initiation signals in natural mRNA involve more than an exposed AUG triplet, and that IF3 probably plays a major role in recognition of specific initiation signals either directly or through interaction with the 30*S* ribosomal subunit. These considerations have prompted a search for specific initiation signals at the ribosome binding sites of phage RNA, either in the form of a specific nucleotide sequence or a specific conformation. As seen in Fig. 2, the nucleotide sequence on either side of the initiator codon is different for each cistron of R17 and Qβ RNA. Although one can readily discern certain short sequences in common among some of the binding sites (e.g., the sequence UUUGA in the C and M sites of R17 RNA and in the C site of Qβ RNA), it would be premature to

designate any of these sequences as a specific initiation signal.

Translational Control by Phage Proteins

In addition to the regulatory role played by RNA conformation, and possibly by ribosomes and initiation factors, a fourth mechanism for translational control at the initiation step has been discovered with the RNA phages, namely, specific binding of proteins to the messenger. Two instances of this phenomenon have been demonstrated in vitro: (i) inhibition of synthetase initiation by phage coat protein and (ii) inhibition of coat protein initiation by the RNA synthetase enzyme complex.

As indicated in an earlier section of this review, in *E. coli* infected with phage coat protein mutants, synthetase formation, and to a much less extent maturation protein formation are enhanced. These observations suggested that, during infection by wild-type phage, coat protein may act as a translational repressor, and this function of viral coat protein has been established in vitro.

When coat protein is incubated with phage RNA and the RNA is then used as a messenger in vitro, the formation of synthetase is specifically inhibited (30, 151, 184–186, 206, 207). This inhibition occurs at the initiation step, as shown by the dipeptide assay (106, 154, 166). Exogenous coat protein does not, in contrast, affect either the initiation or elongation of coat protein (30, 106, 114, 185). As for the effect of coat protein on synthesis of maturation protein, it has been clearly shown that initiation of maturation protein is unaffected (106). The possibility that coat protein inhibits some stage in the elongation of maturation protein has been suggested (30, 95, 207), but the most direct experiments indicate that formation of acid-precipitable polypeptides yielding the *N*-terminal tryptic peptide of maturation protein is the same in the presence or absence of exogenous coat protein (114).

The effect of coat protein on translation of phage RNA is correlated with formation of a protein-RNA complex, which has been called complex I (183). Such a complex, first detected among the products of in vitro phage protein synthesis (13, 16), is maximally formed upon incubation of coat protein (isolated by extraction of virus particles with acetic acid) and phage RNA in a molar ratio of about 10 to 1. [A different complex, resembling phage particles, is formed at ratios greater than 50 molecules of coat protein per RNA molecule (183).] The stability and composition of complex I vary with ionic conditions (182). In 0.1 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride at *p*H 7.4, about six molecules of coat protein are bound per RNA molecule at saturation (30, 183), whereas, in the presence of 0.01 M magnesium acetate, 0.08 M KCl, and 0.01 M Tris at *p*H 7.5, only one molecule of coat protein is tightly bound (167). With RNA fragments made with ribonuclease IV of *E. coli*, it was shown that the coat protein-binding site is within the 3′ 60% of the molecule, the part which contains the synthetase gene (167); more precise localization of the binding site(s) has not been possible.

The formation of complex I is well correlated with the inhibitory effect of coat protein on formation of RNA synthetase. The coat concentration curves are similar for the two reactions (30), and the specificities of the reactions are similar. For example, coat protein from MS2 or f2 does not form a complex and does not repress with Qβ RNA, nor does Qβ coat protein form a complex or repress with MS2 or f2 RNA (30, 151, 207), although each coat protein is active with its homologous RNA. (In contrast with these results with acetic acid-extracted coat protein, guanidine-extracted coat protein is less specific in its interaction with phage RNA (74).) No complex formation or translational inhibition is observed when phage coat protein is mixed with synthetic polynucleotides or with plant virus RNA (66, 184).

The other phage protein which has been shown to have translational effects in vitro is RNA synthetase. Purified Qβ synthetase (the complex of one virus-specified polypeptide and three host-specified polypeptides) inhibits the formation of an initiation complex between ribosomes, Qβ RNA, and fMet-tRNA$_f$ (91). When synthetase was added to Qβ polysomes in the process of translating the phage RNA, there was release of ribosomes and cessation of protein synthesis, presumably due to inhibition of reinitiation (90). Since these experiments were done with native RNA, the synthetase effect was presumably directed at the coat protein initiation site. Recently this effect of the RNA synthetase complex has been attributed to one of the host polypeptides present in the tetrameric enzyme rather than to the phage-specified subunit. This host component appears to be identical to the i factor (discussed above) which is present in uninfected *E. coli* and inhibits coat protein initiation in vitro (50b). Interestingly, when i factor is associated with the Qβ-specified synthetase subunit, the inhibitory effect of the complex shows marked specificity for Qβ RNA (90),

whereas free i factor isolated from uninfected cells inhibits with MS2 as well as Qβ RNA (50a).

SUMMARY OF TRANSLATIONAL CONTROL MECHANISMS IN INFECTED CELLS

In this section we bring together observations made in infected cells and in cell extracts in an attempt to explain the regulation of synthesis of each phage protein during phage development.

Early Protein Synthesis

As described above, only one phage protein, RNA synthetase, is detectable in infected cells labeled between 5 and 15 min after infection. Since this early synthetase formation occurs normally even in the absence of phage RNA synthesis (e.g., after infection at 43 C with a *ts* synthetase mutant), the enzyme must be translated directly from the infecting RNA molecule, which can be found associated with polysomes within the first 4 min of infection (47). Translation of the synthetase gene is, however, dependent on at least partial translation of the coat protein gene, since su$^-$ cells infected with a polar coat protein mutant do not make detectable synthetase and do not efficiently convert the input RNA to a double-stranded form. Thus, on the infecting RNA template, translation of the coat cistron must proceed far enough to open the synthetase initiation site, but translation of the coat gene is apparently not completed since whole molecules of coat protein are not found (Fig. 6). Elongation of the coat polypeptide beyond a certain point might be prevented by some peculiar feature of the infecting RNA. As has been shown recently, maturation protein enters the cell attached to phage RNA (96, 140), and it is possible that the bound maturation protein inhibits coat polypeptide elongation. This hypothesis should be testable in vitro if a suitable complex can be made. [If maturation protein does play a role in translational control of the infecting RNA, it is clearly not an essential function, since phenol-extracted MS2 RNA is infectious for spheroplasts (179).] An alternative explanation is that the infecting RNA has an unusual conformation which allows coat protein initiation but prevents its elongation (42). No example of a conformational restriction on elongation or termination is known, however. On the contrary, the extensive double-stranded regions present within the coat cistron (Fig. 1) are apparently melted out as the ribosome advances. A third possibility is that translational control factor i inhibits initiation of the coat cistron on the infecting RNA (50a, 50b). This hypothesis, however, is not consistent with the apparent need for translation of the early part of the coat protein cistron in order to initiate at the S site, as observed in cells infected with polar coat protein mutants. Also, in in vitro experiments with native RNA, i factor does not relieve the dependence of synthetase initiation on prior translation of the coat protein cistron (50a). Although the mechanism is as yet unclear, failure to make whole molecules of coat protein during the first few minutes of infection ensures the unrestricted translation of the synthetase gene.

After translation of synthetase from the infecting RNA, the RNA must be used as a template for replication. The recent in vitro finding that one of the host subunits associated with Qβ synthetase inhibits binding of ribosomes to the coat protein initiation site provides a possible mechanism for efficient conversion of the input RNA molecule from a messenger to a template for replication.

Late Protein Synthesis

Around 10 to 20 min after infection, when replicating structures (9, 86) and single-stranded progeny RNA molecules begin to accumulate in infected cells (9, 46), the first molecules of maturation protein and coat protein can be detected (Fig. 5). Shortly thereafter, synthetase formation rapidly tapers off and maturation protein synthesis then diminishes, but coat protein continues to be made at a near maximal rate (Fig. 5).

In regard to the cessation of synthetase formation, the simplest explanation is that newly formed coat protein reaches a sufficiently high concentration in the cell to bind to phage RNA, forming complex I and thus directly inhibiting initiation of synthetase as demonstrated in vitro. The excess synthetase formation seen in cells infected with coat protein mutants is readily explained in these terms, and indeed a complex between coat protein and phage RNA, resembling complex I, has been detected in infected cells (25, 147). An alternative explanation has been proposed, namely, that coat protein binds to replicating RNA molecules, as demonstrated in vitro (151), and inhibits transcription of the 3′ end of the RNA containing the synthetase gene. This hypothesis, however, cannot explain the lack of synthetase formation on completed RNA mol-

ecules which are abundant in the polysome fraction of infected cells (46). Furthermore, recent experiments indicate that the sites on the replicating molecules to which coat protein binds are in the single-stranded tails (89). Direct experiments in which phage RNA, isolated at late times from rifampin-treated infected cells (181) or from phage-specific polysomes (88), was used as messenger in vitro demonstrated that deproteinization of this RNA allows translation of synthetase; therefore, in the infected cell the synthetase gene must have been transcribed and then subsequently blocked at the level of translation by protein bound to the RNA.

In regard to maturation protein synthesis, we need to explain why its maximal rate of formation is much less than that of coat protein and why its synthesis diminishes more rapidly than that of coat protein (Fig. 5). Probably the simplest explanation with experimental support is that the secondary structure of the RNA regulates maturation protein synthesis. In this view, no initiation of maturation protein can occur with intact phage RNA as messenger, as observed in vitro. However, since nascent RNA chains present in replicating structures may have a different conformation which allows relatively efficient initiation at the M site, as observed in vitro, it has been proposed that RI is the principal or only messenger for in vivo maturation protein synthesis (150). As noted earlier, replicating RNA molecules are present in the polysome fraction of infected cells; hence, they do function as messenger. Since cells contain far fewer molecules of RI than single-stranded RNA molecules around 15 to 25 min after infection (9), this would account for the observed disparity in rate of maturation and coat protein synthesis. Furthermore, utilization of RI as the messenger for maturation protein synthesis might explain the observation that production of maturation protein is somewhat elevated in cells infected with nonpolar coat protein mutants, since overproduction of synthetase secondarily leads to excessive accumulation of double-stranded RNA in such cells (115, 127). The observed decrease in rate of maturation protein synthesis midway through the infection in actinomycin- (127) or rifampin-treated cells (41, 181) occurs at about the time that viral RNA synthesis stops (41 and Fig. 5) and probably reflects depletion of functional RI.

Although the above conformational mechanism for control of maturation protein synthesis is best supported by current evidence, other explanations have not been excluded. In particular, it is possible that a low rate of initiation of maturation protein is set by a limiting host component, such as a particular class of ribosomes or a specific initiation factor, an hypothesis suggested by in vitro experiments described earlier. Against this explanation is the observation that the rate of synthesis of maturation protein increases with the amount of phage RNA present in rifampin-treated cells over a 15-fold range of RNA content (Kozak and Nathans, *unpublished data*). Furthermore, when fragmented viral RNA is used as messenger in an *E. coli* cell extract, maturation protein can be initiated just as efficiently as coat protein (Kozak and Nathans, *unpublished data*). Thus, in vitro at least, the putative cistron-specific components of the host translational machinery are not limiting for maturation protein initiation.

In regard to the late synthesis of coat protein, there is no compelling evidence for negative or positive control. As noted earlier, the failure to find incomplete coat polypeptide in cells infected with an amber coat protein mutant has led to the suggestion that functional coat protein is needed for efficient translation of the coat protein gene. However, this effect has not been seen with a *ts* coat protein mutant and clearly does not occur in vitro.

CONCLUSIONS

Although our understanding of translation of the RNA coliphage genome exceeds that for any other natural messenger, many questions remain unanswered. Characteristically, new information, especially nucleotide sequence data in this case, has allowed a deeper level of inquiry. To focus on translational control, it is clear that the initiation reaction is the main regulatory step. Therefore, it is particularly important to define this reaction more precisely. What is the initiation signal around each of the natural initiation codons? What molecules—either initiation factors or ribosomal components—recognize the signals? In regard to the conformation of the messenger, we need to know what actual conformations exist, as opposed to hypothetical models based largely on nucleotide sequence analysis, to understand how folding of the RNA regulates translation. In regard to the function of phage proteins as translational regulators, we do not know precisely where on the RNA any of the phage proteins binds nor whether the protein acts directly at the binding site or by stabilizing a less favored conformation of the RNA.

The small genome of the RNA phage shows remarkable genetic economy in carrying out its

regulatory functions. The phage RNA and proteins have multiple roles. Not only does the RNA serve as template for both translation and replication, but within the molecule there appear to be regions which function as codons and which also contribute to a conformation important in translational regulation. Both coat protein and the synthetase complex have a regulatory function in addition to their function as structural protein and enzyme, respectively, and it is possible that maturation protein also has a similar dual role.

The extent to which findings on the translation of coliphage RNA, and in particular on translational control, will be generally applicable is not known at present. In one case at least, namely polarity, the mechanism which operates in the coliphage system appears to be quite different from that which occurs during translation of some bacterial operons (19, 99, 123, 124). Since it is now possible to synthesize identifiable proteins starting with DNA or specific messenger RNA species of cellular or viral origin, it will be interesting to learn whether the mechanisms worked out largely with coliphage RNA apply also to the translation of mRNA from bacterial and eukaryotic cells. In any case, results obtained with the RNA coliphages have greatly influenced thinking about translational mechanisms and illustrate the advantages of concentrated work on a simple experimental system.

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